

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification⁵ : C07K 15/06, C12N 15/12 A61K 37/02</p>	<p>A1</p>	<p>(11) International Publication Number: WO 92/05199 (43) International Publication Date: 2 April 1992 (02.04.92)</p>
<p>(21) International Application Number: PCT/US91/07069 (22) International Filing Date: 26 September 1991 (26.09.91) (30) Priority data: 588,227 26 September 1990 (26.09.90) US (71) Applicant: GENETICS INSTITUTE, INC. [US/US]; 87 CambridgePark Drive, Cambridge, MA 02140 (US). (72) Inventors: WOZNEY, John, M. ; 59 Old Bolton Road, Hudson, MA 01749 (US). ROSEN, Vicki, A. ; 127 Kilsyth Road, Brookline, MA 02146 (US). WANG, Elizabeth, A. ; 136 Wolf Rock Road, Carlisle, MA 01741 (US). CELESTE, Anthony, J. ; 86 Packard Street, Hudson, MA 01479 (US).</p>		<p>(74) Agents: KAPINOS, Ellen, J. et al.; Genetics Institute, Inc., 87 CambridgePark Drive, Cambridge, MA 02140 (US). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>BEST AVAILABLE COPY</p>
<p>(54) Title: BMP-5 DERIVATIVES (57) Abstract Purified BMP-5 protein derivatives comprising parts of human BMP-5 protein and processes for producing them are disclosed. The proteins may be used in the treatment of bone and/or cartilage defects and in wound healing and related tissue repair.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU ⁺	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE ⁺	Germany	MC	Monaco	US	United States of America
DK	Denmark				

⁺ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

BMP-5 DERIVATIVES

5

The present invention relates to a family of purified proteins, termed BMP-5 proteins (wherein BMP is bone morphogenic protein), which exhibit the ability to induce cartilage and/or bone formation and processes for obtaining them. These proteins may be used to induce bone and/or cartilage formation and in wound healing and tissue repair.

10

15

The invention provides human BMP-5 proteins, substantially free from other proteins with which they are co-produced, comprising the amino acid sequence set forth in Table III from amino acid # 323 (Asn, Gln, Asn) to amino acid # 454 (ending with Gly, Cys, His). This amino acid sequence #323 to #454 is encoded by the DNA sequence of Table III from nucleotide # 1665 to nucleotide # 2060. The mature BMP-5 dimer may be further characterized by an apparent molecular weight of approximately 28,000-38,000 daltons as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Under reducing conditions in SDS-PAGE the mature subunit electrophoreses with a molecular weight of approximately 18,000 - 22,000 daltons. These proteins are capable of stimulating, promoting, or otherwise inducing cartilage and/or bone formation.

20

25

30

35

The invention further provides bovine BMP-5 proteins comprising the amino acid sequence set forth in Table I from #9 to amino acid #140. The amino acid sequence from #9 to #140 is encoded by the DNA sequence from nucleotide # 32 to #427 of Table I. These proteins may be further characterized by an apparent molecular weight of 28,000 - 30,000 daltons as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Under reducing conditions in SDS-PAGE the protein electrophoreses with a molecular weight of approximately 14,000-20,000 daltons. It is contemplated that these proteins are capable of inducing cartilage and/or bone formation.

2.

Human BMP-5 proteins of the invention may be produced by culturing a cell transformed with a DNA sequence containing the nucleotide sequence the same or substantially the same as the nucleotide sequence shown in Table III comprising nucleotide # 699 to nucleotide # 2060. BMP-5 proteins comprising the amino acid sequence the same or substantially the same as shown in Table III from amino acid #323 to #454 are recovered isolated and purified from the culture media.

Bovine proteins of the invention may be produced by culturing a cell transformed with a DNA sequence containing the nucleotide sequence the same or substantially the same as that shown in Table I comprising nucleotide #8 through nucleotide #427 and recovering and purifying from the culture medium a protein containing the amino acid sequence or a portion thereof as shown in Table I comprising amino acid #9 to amino acid #140.

The invention further provides a method wherein the proteins described above are utilized for obtaining related human protein/s or other mammalian cartilage and/or bone growth protein/s. Such methods are known to those skilled in the art of genetic engineering. One method for obtaining such proteins involves utilizing the human BMP-5 coding sequence or portions thereof from nucleotide # 699 - # 2060 as a probe for screening human genomic and/or cDNA libraries to isolate the human genomic and/or cDNA sequence. Additional methods known in the art may employ the bovine and human BMP-5 proteins of the invention to obtain other mammalian BMP-5 cartilage and/or bone formation proteins.

Having identified the nucleotide sequences the proteins are produced by culturing a cell transformed with the DNA identified in the method described above which DNA hybridizes under stringent conditions to the bovine BMP-5 nucleotide sequence substantially as shown in Table I or the human BMP-5 nucleotide sequence substantially as shown in Table III and which encodes a protein exhibiting cartilage and/or bone formation activity. The expressed proteins are recovered and purified from the culture media. The purified BMP-5 proteins are substantially free from other

proteinaceous materials with which they are co-produced, as well as from other contaminants. The BMP-5 proteins of the invention are characterized by the ability to promote, stimulate or otherwise induce the formation of cartilage and/or bone. It is further contemplated that the ability of these proteins to induce the formation of cartilage and/or bone may be exhibited by the ability to demonstrate cartilage and/or bone formation activity in the rat bone formation assay described below. It is further contemplated that the proteins of the invention may demonstrate activity in this rat bone formation assay at a concentration of $10\mu\text{g}$ - $500\mu\text{g}$ /gram of bone. More particularly, it is contemplated these proteins may be characterized by the ability of $1\mu\text{g}$ of the protein to score at least +2 in the rat bone formation assay described below using either the original or modified scoring method.

Another aspect of the invention provides pharmaceutical compositions containing a therapeutically effective amount of a BMP-5 protein of the invention in a pharmaceutically acceptable vehicle or carrier. The compositions of the invention may be used to induce bone and/or cartilage formation. These compositions may also be used for wound healing and tissue repair. Further compositions of the invention may include in addition to a BMP-5 protein of the present invention at least one other therapeutically useful agent such as the proteins designated BMP-1, BMP-2A and -2B, BMP-3, BMP-6, and BMP-7 disclosed respectively in co-owned U.S. patent applications Serial No. 179,101, Serial No. 179,100, and Serial No. 179,197, Serial No. 370,544, and Serial No. 370,549. These proteins may act in concert with or perhaps synergistically with one another. Other therapeutically useful agents may include growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factors ($\text{TGF-}\alpha$ and $\text{TGF-}\beta$), and platelet derived growth factor (PDGF).

The compositions of the invention may also include an appropriate matrix, for instance, for delivery and/or support of the composition and/or providing a surface for bone and/or cartilage formation. The matrix may provide slow release of the

BMP-5 proteins and/or the appropriate environment for presentation of the BMP-5 proteins of the invention.

The compositions may be employed in methods for treating a number of bone and/or cartilage defects, and periodontal disease. They may also be employed in methods for treating various types of wounds and in tissue repair. These methods, according to the invention, entail administering to a patient needing such bone and/or cartilage formation, wound healing or tissue repair, a therapeutically effective amount of a protein of the invention. These methods may also entail the administration of a protein of the invention in conjunction with at least one of the "BMP" proteins disclosed in the co-owned applications described above. In addition, these methods may also include the administration of a protein of the invention with other growth factors including EGF, FGF, TGF- α , TGF- β , and PDGF.

Still a further aspect of the invention are DNA sequences coding for expression of a protein of the invention. Such sequences include the sequence of nucleotides in a 5' to 3' direction illustrated in Table I or Table III or DNA sequences which hybridize under stringent conditions with the DNA sequence of Table I or Table III and encode a protein demonstrating ability to induce cartilage and/or bone formation as in the rat bone formation assay described below. It is contemplated that these proteins may demonstrate activity in this assay at a concentration of 10 μ g - 500 μ g/gram of bone. More particularly, it is contemplated that these proteins demonstrate the ability of 1 μ g of the protein to score at least +2 in the rat bone formation assay using either the original or modified scoring method. Finally, allelic or other variations of the sequences of Table I and III whether such nucleotide changes result in changes in the peptide sequence or not, are also included in the present invention.

A further aspect of the invention provides a vector containing a DNA sequence as described above in operative association with an expression control sequence therefor. These vectors may be employed in a novel process for producing a protein of the

invention in which a cell line transformed with a DNA sequence directing expression of a protein of the invention in operative association with an expression control sequence therefor, is cultured in a suitable culture medium and a protein of the invention is recovered and purified therefrom. This claimed process may employ a number of known cells, both prokaryotic and eukaryotic, as host cells for expression of the polypeptide. The recovered BMP proteins are purified by isolating them from other proteinaceous materials with which they are co-produced as well as from other contaminants.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description and preferred embodiments thereof.

Detailed Description of the Invention

Purified human BMP-5 proteins are produced by culturing a host cell transformed with the DNA sequence of Table III. The expressed BMP-5 proteins are isolated and purified from the culture media. The purified human BMP-5 proteins are characterized by comprising an amino acid sequence as shown in Table III from amino acid #323 to #454. These purified BMP-5 human cartilage/bone proteins of the present invention may be produced by culturing a host cell transformed with a DNA sequence comprising the DNA sequence as shown in Table III from nucleotide # 699 to nucleotide # 2060 or substantially homologous sequences operatively linked to a heterologous regulatory control sequence and recovering and purifying from the culture medium a protein comprising the amino acid sequence as shown in Table III from amino acid # 323 to amino acid # 454 or a substantially homologous sequence.

In further embodiments the DNA sequence comprises the nucleotides encoding amino acids #323-#454 of Table III. BMP-5 proteins may therefore be produced by culturing a host cell transformed with a DNA sequence comprising the DNA sequence as shown in Table III from nucleotide # 1665 to nucleotide # 2060 or substantially homologous sequences operatively linked to a

heterologous regulatory control sequence and recovering and purifying from the culture medium a protein comprising the amino acid sequence as shown in Table III from amino acid # 323 to amino acid # 454 or a substantially homologous sequence. The purified human BMP-5 proteins are substantially free from other proteinaceous materials with which they are co-produced, as well as from other contaminants.

Purified BMP-5 bovine cartilage/bone proteins of the present invention are produced by culturing a host cell transformed with a DNA sequence comprising the DNA sequence as shown in Table I from nucleotide # 8 to nucleotide # 578 or substantially homologous sequences and recovering and purifying from the culture medium a protein comprising the amino acid sequence as shown in Table I from amino acid # 9 to amino acid # 140 or a substantially homologous sequence. The purified BMP-5 bovine proteins of the invention are substantially free from other proteinaceous materials with which they are co-produced, as well as from other contaminants.

BMP-5 proteins are further characterized by the ability to demonstrate cartilage and/or bone formation activity. This activity may be demonstrated, for example, in the rat bone formation assay as described in Example III. It is further contemplated that these proteins demonstrate activity in the assay at a concentration of $10\mu\text{g}$ - $500\mu\text{g}$ /gram of bone formed. The proteins may be further characterized by the ability of $1\mu\text{g}$ to score at least +2 in this assay using either the original or modified scoring method described below.

The mature BMP-5 dimer may be further characterized by an apparent molecular weight of approximately 28,000-38,000 daltons as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Under reducing conditions in SDS-PAGE the mature sub-unit electrophoreses with a molecular weight of approximately 18,000 - 22,000 daltons.

The proteins provided herein also include factors encoded by the sequences similar to those of Table I and Table III but into which modifications are naturally provided (e.g. allelic variations

in the nucleotide sequence which may result in amino acid changes in the polypeptide) or deliberately engineered. Similarly, synthetic polypeptides which wholly or partially duplicate continuous sequences of the amino acid residues of Table I or Table III are encompassed by the invention. These sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with other cartilage/bone proteins of the invention may possess bone and/or cartilage growth factor biological properties in common therewith. Thus, they may be employed as biologically active substitutes for naturally-occurring proteins in therapeutic processes.

Other specific mutations of the sequences of the proteins of the invention described herein may involve modifications of a glycosylation site. These modification may involve O-linked or N-linked glycosylation sites. For instance, the absence of glycosylation or only partial glycosylation at asparagine-linked glycosylation sites results from amino acid substitution or deletion at any asparagine-linked glycosylation recognition site present in the sequences of the proteins of the invention, for example, as shown in Table I or Table III. The asparagine-linked glycosylation recognition sites comprise tripeptide sequences which are specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either asparagine-X-threonine or asparagine-X-serine, where X is usually any amino acid. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence. Expression of such altered nucleotide sequences produces variants which are not glycosylated at that site.

The present invention also encompasses the novel DNA sequences, free of association with DNA sequences encoding other proteinaceous materials, and coding on expression for the proteins of the invention. These DNA sequences include those depicted in Tables I and III in a 5' to 3' direction. Further included are

those sequences which hybridize under stringent hybridization conditions [see, T. Maniatis et al, Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pages 387 to 389] to the DNA sequence of Table I or Table III and demonstrate cartilage and/or bone formation activity in the rat bone formation assay. An example of one such stringent hybridization condition is hybridization at 4 x SSC at 65°C, followed by a washing in 0.1 x SCC at 65°C for an hour. Alternatively, an exemplary stringent hybridization condition is in 50% formamide, 4 x SCC at 42°C.

Similarly, DNA sequences which encode proteins similar to the protein encoded by the sequence of Table I or Table III, but which differ in codon sequence due to the degeneracies of the genetic code or allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) also encode the proteins of the invention described herein. Variations in the DNA sequences of Table I and Table III which are caused by point mutations or by induced modifications (including insertion, deletion, and substitution) to enhance the activity, half-life or production of the polypeptides encoded thereby are also encompassed in the invention.

In a further aspect, the invention provides a method for obtaining related human proteins or other mammalian BMP-5 proteins. One method for obtaining such proteins entails, for instance, utilizing the human BMP-5 coding sequence disclosed herein to probe a human genomic library using standard techniques for the human gene or fragments thereof. Sequences thus identified may also be used as probes to identify a human cell line or tissue which synthesizes the analogous cartilage/bone protein. A cDNA library is synthesized and screened with probes derived from the human or bovine coding sequences. The human sequence thus identified is transformed into a host cell, the host cell is cultured and the protein recovered, isolated and purified from the culture medium. The purified protein is predicted to exhibit cartilage and/or bone formation activity in the rat bone formation assay of Example III.

Another aspect of the present invention provides a novel

method for producing the proteins of the invention. The method of the present invention involves culturing a suitable cell line, which has been transformed with a DNA sequence coding for expression of a protein of the invention, under the control of known regulatory sequences. Regulatory sequences include promoter fragments, terminator fragments and other suitable sequences which direct the expression of the protein in an appropriate host cell. Methods for culturing suitable cell lines are within the skill of the art. The transformed cells are cultured and the BMP-5 proteins expressed thereby are recovered and purified from the culture medium using purification techniques known to those skilled in the art. The purified BMP-5 proteins are substantially free from other proteinaceous materials with which they are co-produced, as well as other contaminants. Purified BMP-5 proteins are substantially free from materials with which the proteins of the invention exist in nature.

— Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al, U.S. Patent 4,419,446. Other suitable mammalian cell lines are the monkey COS-1 cell line and the CV-1 cell line.

Bacterial cells may also be suitable hosts. For example, the various strains of E. coli (e.g., HB101, MC1061) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art may also be available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g. Miller et al, Genetic

Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

Another aspect of the present invention provides vectors for use in the method of expression of the proteins of the invention. The vectors contain the novel DNA sequences described above which code for the novel cartilage/bone proteins of the invention. Additionally the vectors also contain appropriate expression control sequences permitting expression of the protein sequences. Alternatively, vectors incorporating truncated or modified sequences as described above are also embodiments of the present invention and useful in the production of the proteins of the invention. The vectors may be employed in the method of transforming cell lines and contain selected regulatory sequences in operative association with the DNA coding sequences of the invention which are capable of directing the replication and expression thereof in selected host cells. Useful regulatory sequences for such vectors are known to those skilled in the art and may be selected depending upon the selected host cells. Such selection is routine and does not form part of the present invention. Host cells transformed with such vectors and progeny thereof for use in producing cartilage/bone proteins are also provided by the invention.

One skilled in the art can construct mammalian expression vectors by employing the DNA sequences of the invention and known vectors, such as pCD [Okayama et al., Mol. Cell Biol., 2:161-170 (1982)] and pJL3, pJL4 [Gough et al., EMBO J., 4:645-653 (1985)].

Similarly, one skilled in the art could manipulate the sequences of the invention by eliminating or replacing the mammalian regulatory sequences flanking the coding sequence with bacterial sequences to create bacterial vectors for intracellular or extracellular expression by bacterial cells. For example, the coding sequences could be further manipulated (e.g. ligated to other known linkers or modified by deleting non-coding sequences therefrom or altering nucleotides therein by other known techniques). The modified coding sequence could then be inserted

into a known bacterial vector using procedures such as described in T. Taniguchi et al., Proc. Natl Acad. Sci. USA, 77:5230-5233 (1980). This exemplary bacterial vector could then be transformed into bacterial host cells and a protein of the invention expressed thereby. For a strategy for producing extracellular expression of a cartilage and/or bone protein of the invention in bacterial cells., see, e.g. European patent application EPA 177,343.

Similar manipulations can be performed for the construction of an insect vector [See, e.g. procedures described in published European patent application 155,476] for expression in insect cells. A yeast vector could also be constructed employing yeast regulatory sequences for intracellular or extracellular expression of the factors of the present invention by yeast cells. [See, e.g., procedures described in published PCT application WO86/00639 and European patent application EPA 123,289].

A method for producing high levels of a protein of the invention from mammalian cells involves the construction of cells containing multiple copies of the heterologous gene encoding proteins of the invention. The heterologous gene may be linked to an amplifiable marker, e.g. the dihydrofolate reductase (DHFR) gene for which cells containing increased gene copies can be selected for propagation in increasing concentrations of methotrexate (MTX) according to the procedures of Kaufman and Sharp, J. Mol. Biol., 159:601-629 (1982). This approach can be employed with a number of different cell types.

For instance, a plasmid containing a DNA sequence for a protein of the invention in operative association with other plasmid sequences enabling expression thereof and the DHFR expression plasmid pAdA26SV(A)3 [Kaufman and Sharp, Mol. Cell. Biol., 2:1304 (1982)] may be co-introduced into DHFR-deficient CHO cells, DUKX-BII, by calcium phosphate coprecipitation and transfection, electroporation or protoplast fusion. DHFR expressing transformants are selected for growth in alpha media with dialyzed fetal calf serum, and subsequently selected for amplification by growth in increasing concentrations of MTX

(sequential steps in 0.02, 0.2, 1.0 and 5uM MTX) as described in Kaufman et al., Mol Cell Biol., 5:1750 (1983). Protein expression should increase with increasing levels of MTX resistance. Transformants are cloned, and the proteins of the invention are recovered, isolated, and purified from the culture medium. Characterization of expressed proteins may be carried out using standard techniques. For instance, characterization may include pulse labeling with [³⁵S] methionine or cysteine, or polyacrylamide gel electrophoresis. Biologically active protein expression is monitored by the Rosen-modified Sampath - Reddi rat bone formation assay described above in Example III. Similar procedures can be followed to produce other related proteins.

A protein of the present invention, which induces cartilage and/or bone formation in circumstances where bone and/or cartilage is not normally formed, has application in the healing of bone fractures and cartilage defects in humans and other animals. Such a preparation employing a protein of the invention may have prophylactic use in closed as well as open fracture reduction and also in the improved fixation of artificial joints. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery. A protein of the invention may be used in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. A variety of osteogenic, cartilage-inducing and bone inducing factors have been described. See, e.g. European patent applications 148,155 and 169,016 for discussions thereof.

The proteins of the invention may also be used in wound healing and related tissue repair. The types of wounds include, but are not limited to burns, incisions and ulcers. (See, e.g. PCT Publication WO84/01106 for discussion of wound healing and related tissue repair).

A further aspect of the invention includes a therapeutic method and composition for repairing fractures and other conditions related to bone and/or cartilage defects or periodontal diseases. In addition, the invention comprises therapeutic methods and compositions for wound healing and tissue repair. Such compositions comprise a therapeutically effective amount of at least one of the BMP-5 proteins of the invention in admixture with a pharmaceutically acceptable vehicle, carrier or matrix.

It is expected that the proteins of the invention may act in concert with or perhaps synergistically with one another or with other related proteins and growth factors. Therapeutic methods and compositions of the invention therefore comprise one or more of the proteins of the present invention. Further therapeutic methods and compositions of the invention therefore comprise a therapeutic amount of at least one protein of the invention with a therapeutic amount of at least one of the other "BMP" proteins, BMP-1, BMP-2 (~~BMP-2A, BMP-2 Class I~~), ~~BMP-3, BMP-4~~ (~~BMP-2B, BMP-2 Class II~~), BMP-6, and BMP-7 disclosed in co-owned and co-pending U.S. applications described above. Such methods and compositions of the invention may comprise proteins of the invention or portions thereof in combination with the above-mentioned "BMP" proteins or portions thereof. Such combination may comprise individual separate molecules from each of the proteins or heteromolecules such as heterodimers formed by portions of the respective proteins. For example, a method and composition of the invention may comprise a protein of the invention or a portion thereof linked with a portion of a "BMP" protein to form a heteromolecule.

Further therapeutic methods and compositions of the invention comprise the proteins of the invention or portions thereof in combination with other agents beneficial to the treatment of the bone and/or cartilage defect, wound, or tissue in question. These agents include various growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), transforming growth factors (TGF- α and TGF- β), k-fibroblast growth factor (kFGF), parathyroid hormone (PTH),

leukemia inhibitory factor (LIF/HILDA DIA) and insulin-like growth factors (IGF-I and IGF-II). Portions of these agents may also be used in compositions of the invention.

5 The preparation and formulation of such physiologically acceptable protein compositions, having due regard to pH, isotonicity, stability and the like, is within the skill of the art. The therapeutic compositions are also presently valuable for veterinary applications due to the apparent lack of species
10 specificity in cartilage and bone growth factor proteins. Domestic animals and thoroughbred horses in addition to humans are desired patients for such treatment with the proteins of the present invention.

The therapeutic method includes administering the composition topically, systematically, or locally as an implant or device.
15 When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of cartilage and/or bone or tissue damage. Topical administration
20 may be suitable for wound healing and tissue repair. Preferably for bone and/or cartilage formation, the composition would include a matrix capable of delivering the cartilage/bone proteins of the invention to the site of bone and/or cartilage damage, providing a structure for the developing bone and cartilage and optimally
25 capable of being resorbed into the body. The matrix may provide slow release of the BMP proteins or other factors comprising the composition. Such matrices may be formed of materials presently in use for other implanted medical applications.

The choice of matrix material is based on biocompatibility,
30 biodegradability, mechanical properties, cosmetic appearance and interface properties. The particular application of the compositions of the invention will define the appropriate formulation. Potential matrices for the compositions may be biodegradable and chemically defined calcium sulfate,
35 tricalciumphosphate, hydroxyapatite, polylactic acid and

polyanhydrides. Other potential materials are biodegradable and biologically well defined, such as bone or dermal collagen. Further matrices are comprised of pure proteins or extracellular matrix components. Other potential matrices are nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics. Matrices may be comprised of combinations of any of the above mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate and processing to alter pore size, particle size, particle shape, and biodegradability.

The dosage regimen will be determined by the attending physician considering various factors which modify the action of the proteins of the invention. Factors which may modify the action of the proteins of the invention include the amount of bone weight desired to be formed, the site of bone damage, the condition of the damaged bone, the size of a wound, type of damaged tissue, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the type or types of bone and/or cartilage proteins present in the composition. The addition of other known growth factors, such as EGF, PDGF, TGF- α , TGF- β , and IGF-I to the final composition, may also effect the dosage.

Progress can be monitored by periodic assessment of cartilage and/or bone growth and/or repair. The progress can be monitored, for example, using x-rays, histomorphometric determinations and tetracycline labeling .

The following examples illustrate practice of the present invention in recovering and characterizing bovine cartilage and/or bone proteins of the invention and employing these proteins to recover the corresponding human protein or proteins and in expressing the proteins via recombinant techniques.

EXAMPLE I

Isolation of Bovine Cartilage/Bone Inductive Protein

Ground bovine bone powder (20-120 mesh, Helitrex) is prepared according to the procedures of M. R. Urist et al., Proc. Natl Acad. Sci USA, 70:3511 (1973) with elimination of some extraction steps as identified below. Ten kgs of the ground powder is demineralized in successive changes of 0.6N

HCl at 4°C over a 48 hour period with vigorous stirring. The resulting suspension is extracted for 16 hours at 4°C with 50 liters of 2M CaCl₂ and 10mM ethylenediamine-tetraacetic acid [EDTA], and followed by extraction for 4 hours in 50 liters of 0.5M EDTA. The residue is washed three times with distilled water before its resuspension in 20 liters of 4M guanidine hydrochloride [GuCl], 20mM Tris (pH 7.4), 1mM N-ethylmaleimide, 1mM iodoacetamide, 1mM phenylmethylsulfonyl fluorine as described in Clin. Orthop. Rel. Res., 171: 213 (1982). After 16 to 20 hours the supernatant is removed and replaced with another 10 liters of GuCl buffer. The residue is extracted for another 24 hours.

The crude GuCl extracts are combined, concentrated approximately 20 times on a Pellicon apparatus with a 10,000 molecular weight cut-off membrane, and then dialyzed in 50mM Tris, 0.1M NaCl, 6M urea (pH 7.2), the starting buffer for the first column. After extensive dialysis the protein is loaded on a 4 liter DEAE cellulose column and the unbound fractions are collected.

The unbound fractions are concentrated and dialyzed against 50mM NaAc, 50mM NaCl (pH 4.6) in 6M urea. The unbound fractions are applied to a carboxymethyl cellulose column. Protein not bound to the column is removed by extensive washing with starting buffer, and the material containing protein having bone and/or cartilage formation activity as measured by the Rosen-modified Sampath - Reddi assay (described in Example III below) desorbed from the column by 50mM NaAc, 0.25mM NaCl, 6M urea (pH 4.6). The protein from this step elution is concentrated 20- to 40- fold, then diluted 5 times with 80mM KPO₄, 6M urea (pH 6.0). The pH of the

WU 92/05177
10/03/77/000

solution is adjusted to 6.0 with 500mM K_2HPO_4 . The sample is applied to an hydroxylapatite column (LKB) equilibrated in 80mM KPO_4 , 6M urea (pH6.0) and all unbound protein is removed by washing the column with the same buffer. Protein having bone and/or cartilage formation activity is eluted with 100mM KPO_4 (pH7.4) and 6M urea.

The protein is concentrated approximately 10 times, and solid NaCl added to a final concentration of 0.15M. This material is applied to a heparin - Sepharose column equilibrated in 50mM KPO_4 , 150mM NaCl, 6M urea (pH7.4). After extensive washing of the column with starting buffer, a protein with bone and/or cartilage inductive activity is eluted by 50mM KPO_4 , 700mM NaCl, 6M urea (pH7.4). This fraction is concentrated to a minimum volume, and 0.4ml aliquots are applied to Superose 6 and Superose 12 columns connected in series, equilibrated with 4M GuCl, 20mM Tris (pH7.2) and the columns developed at a flow rate of 0.25ml/min. The protein demonstrating bone and/or cartilage inductive activity corresponds to an approximate 30,000 dalton protein.

The above fractions from the superose columns are pooled, dialyzed against 50mM NaAc, 6M urea (pH4.6), and applied to a Pharmacia MonoS HR column. The column is developed with a gradient to 1.0M NaCl, 50mM NaAc, 6M urea (pH4.6). Active bone and/or cartilage formation fractions are pooled. The material is applied to a 0.46 x 25cm Vydac C4 column in 0.1% TFA and the column developed with a gradient to 90% acetonitrile, 0.1% TFA (31.5% acetonitrile, 0.1% TFA to 49.5% acetonitrile, 0.1% TFA in 60 minutes at 1ml per minute). Active material is eluted at approximately 40-44% acetonitrile. Fractions were assayed for cartilage and/or bone formation activity. The active material from the C4 reverse phase column is further fractionated on a MonoQ column. The protein is dialyzed against 6M urea, 25mM diethanolamine, pH 8.6 and then applied to a 0.5 by 5 cm MonoQ column (Pharmacia) which is developed with a gradient of 6M urea, 25mM diethanolamine, pH 8.6 and 0.5 M NaCl, 6M urea, 25mM

diethanolamine, pH 8.6. Fractions are brought to pH3.0 with 10% trifluoroacetic acid (TFA).

Aliquots of the appropriate fractions are iodinated by one of the following methods: P. J. McConahey et al, Int. Arch. Allergy, 29:185-189 (1966); A. E. Bolton et al, Biochem J., 133:529 (1973); and D. F. Bowen-Pope, J. Biol. Chem., 237:5161 (1982). The iodinated proteins present in these fractions are analyzed by SDS gel electrophoresis.

EXAMPLE II

Characterization of Bovine Cartilage/Bone Inductive Factor

A. Molecular Weight

Approximately 5 μ g protein from Example I in 6M urea, 25mM diethanolamine, pH 8.6, approximately 0.3 M NaCl is made 0.1% with respect to SDS and dialyzed against 50 mM tris/HCl 0.1% SDS pH 7.5 for 16 hrs. The dialyzed material is then electrophoretically concentrated against a dialysis membrane [Hunkapillar et al Meth. Enzymol. 91: 227-236 (1983)] with a small amount of I 125 labelled counterpart. This material (volume approximately 100 μ l) is loaded onto a 12% polyacrylamide gel and subjected to SDS-PAGE [Laemmli, U.K. Nature, 227:680-685 (1970)] without reducing the sample with dithiothreitol. The molecular weight is determined relative to prestained molecular weight standards (Bethesda Research Labs). Following autoradiography of the unfixed gel the approximate 28,000-30,000 dalton band is excised and the protein electrophoretically eluted from the gel (Hunkapillar et al supra). Based on similar purified bone fractions as described in the co-pending "BMP" applications described above wherein bone and/or cartilage activity is found in the 28,000-30,000 region, it is inferred that this band comprises bone and/or cartilage inductive fractions.

B. Subunit Characterization

The subunit composition of the isolated bovine bone protein is also determined. The eluted protein described above is fully

reduced and alkylated in 2% SDS using iodoacetate and standard procedures and reconcentrated by electrophoretic packing. The fully reduced and alkylated sample is then further submitted to SDS-PAGE on a 12% gel and the resulting approximate 14,000-20,000 dalton region having a doublet appearance located by autoradiography of the unfixed gel. A faint band remains at the 28,000-30,000 region. Thus the 28,000-30,000 dalton protein yields a broad region of 14,000-20,000 which may otherwise also be interpreted and described as comprising two broad bands of approximately 14,000-16,000 and 16,000-20,000 daltons.

EXAMPLE III

Rosen Modified Sampath-Reddi Assay

A modified version of the rat bone formation assay described in Sampath and Reddi, Proc. Natl. Acad. Sci. U.S.A., 80:6591-6595 (1983) ~~is used to evaluate bone and/or cartilage activity of the proteins of the invention.~~ This modified assay is herein called the Rosen-modified Sampath-Reddi assay. The ethanol precipitation step of the Sampath-Reddi procedure is replaced by dialyzing (if the composition is a solution) or diafiltering (if the composition is a suspension) the fraction to be assayed against water. The solution or suspension is then redissolved in 0.1 % TFA, and the resulting solution added to 20mg of rat matrix. A mock rat matrix sample not treated with the protein serves as a control. This material is frozen and lyophilized and the resulting powder enclosed in #5 gelatin capsules. The capsules are implanted subcutaneously in the abdominal thoracic area of 21 - 49 day old male Long Evans rats. The implants are removed after 5 - 21 days. Half of each implant is used for alkaline phosphatase analysis [See, A. H. Reddi et al., Proc. Natl Acad Sci., 69:1601 (1972)].

The other half of each implant is fixed and processed for histological analysis. Glycolmethacrylate sections (1 μ m) are stained with Von Kossa and acid fuschin or toluidine blue to score the amount of induced bone and cartilage formation present in each

5 implant. The terms +1 through +5 represent the area of each
 histological section of an implant occupied by new bone and/or
 cartilage cells and newly formed bone and matrix. Two scoring
 methods are herein described. The first describes the original
 10 scoring method while the second describes the later adopted scoring
 method. A score of +5 indicates that greater than 50% of the
 implant is new bone and/or cartilage produced as a direct result of
 protein in the implant. A score of +4, +3, +2 and +1 would
 15 indicate that greater than 40%, 30%, 20% and 10% respectively of
 the implant contains new cartilage and/or bone. The scoring method
 later adopted (which hereinafter may be referred to as the
 "modified" scoring method) is as follows: Three non-adjacent
 sections are evaluated from each implant and averaged. "+/-"
 indicates tentative identification of cartilage or bone; "+1"
 20 indicates >10% of each section being new cartilage or bone; "+2",
 >25%; "+3", >50%; "+4", ~75%; "+5", >80%. A "-" indicates that the
 implant is not recovered. The scores of the individual implants
 are tabulated to indicate assay variability. I t i s
 contemplated that the dose response nature of the cartilage and/or
 bone inductive protein containing samples of the matrix samples
 will demonstrate that the amount of bone and/or cartilage formed
 increases with the amount of cartilage/bone inductive protein in
 25 the sample. It is contemplated that the control samples will not
 result in any bone and/or cartilage formation.

30 As with other cartilage and/or bone inductive proteins such as
 the above-mentioned "BMP" proteins, the bone and/or cartilage
 formed is expected to be physically confined to the space occupied
 by the matrix. Samples are also analyzed by SDS gel
 electrophoresis and isoelectric focusing followed by autoradiog-
 35 raphy. The activity is correlated with the protein bands and pI.
 To estimate the purity of the protein in a particular fraction an
 extinction coefficient of 1 OD/mg-cm is used as an estimate for
 protein and the protein is run on SDS-PAGE followed by silver
 staining or radioiodination and autoradiography.

EXAMPLE IVBovine BMP-5 Protein Composition

The gel slice of the approximate 14,000-20,000 dalton region described in Example IIB is fixed with methanol-acetic acid-water using standard procedures, briefly rinsed with water, then neutralized with 0.1M ammonium bicarbonate. Following dicing the gel slice with a razor blade, the protein is digested from the gel matrix by adding 0.2 μ g of TPCK-treated trypsin (Worthington) and incubating the gel for 16 hr. at 37 degrees centigrade. The resultant digest is then subjected to RPHPLC using a C4 Vydac RPHPLC column and 0.1% TFA-water 0.1% TFA water-acetonitrile gradient. The resultant peptide peaks were monitored by UV absorbance at 214 and 280 nm and subjected to direct amino terminal amino acid sequence analysis using an Applied Biosystems gas phase sequenator (Model 470A). One tryptic fragment is isolated by standard procedures having the following amino acid sequence as represented by the amino acid standard three-letter symbols and where "Xaa" indicates an unknown amino acid the amino acid in parentheses indicates uncertainty in the sequence:

Xaa-His-Glu-Leu-Tyr-Val-Ser-Phe-(Ser)

The following four oligonucleotide probes are designed on the basis of the amino acid sequence of the above-identified tryptic fragment and synthesized on an automated DNA synthesizer.

PROBE #1: GTRCTYGANATRCANTC

PROBE #2: GTRCTYGANATRCANAG

PROBE #3: GTRCTYAAAYATRCANTC

PROBE #4: GTRCTYAAAYATRCANAG

The standard nucleotide symbols in the above identified probes are as follows: A,adenine; C,cytosine; G,guanine; T,thymine; N,

adenine or cytosine or guanine or thymine; R, adenine or guanine; and Y, cytosine or thymine.

Each of the probes consists of pools of oligonucleotides. Because the genetic code is degenerate (more than one codon can code for the same amino acid), a mixture of oligonucleotides is synthesized that contains all possible nucleotide sequences encoding the amino acid sequence of the tryptic. These probes are radioactively labeled and employed to screen a bovine cDNA library as described below.

Poly(A) containing RNA is isolated by oligo(dT) cellulose chromatography from total RNA isolated from fetal bovine bone cells by the method of Gehron-Robey et al in Current Advances in Skeletogenesis, Elsevier Science Publishers (1985). The total RNA was obtained from Dr. Marion Young, National Institute of Dental Research, National Institutes of Health. A cDNA library is made in lambda gt10 (Toole et al supra) and plated on 50 plates at 8000 recombinants per plate. These recombinants (400,000) are screened on duplicate nitrocellulose filters with a combination of Probes 1, 2, 3, and 4 using the Tetramethylammonium chloride (TMAC) hybridization procedure [see Wozney et al Science, 242: 1528-1534 (1988)]. Twenty-eight positives are obtained and are replated for secondaries. Duplicate nitrocellulose replicas again are made. One set of filters are screened with Probes 1 and 2; the other with Probes 3 and 4. Six positives are obtained on the former, 21 positives with the latter. One of the six, called HEL5, is plaque purified, a phage plate stock made, and bacteriophage DNA isolated. This DNA is digested with EcoRI and subcloned into M13 and pSP65 (Promega Biotec, Madison, Wisconsin) [Melton, et al., Nucl. Acids Res. 12:7035-7056 (1984)]. The DNA sequence and derived amino acid sequence of this fragment is shown in Table I.

DNA sequence analysis of this fragment in M13 indicates that it encodes the desired tryptic peptide sequence set forth above, and this derived amino acid sequence is preceded by a basic residue (Lys) as predicted by the specificity of trypsin. The underlined portion of the sequence in Table I from amino acid #42 to #48

corresponds to the tryptic fragment identified above from which the oligonucleotide probes are designed. The derived amino acid sequence Ser-Gly-Ser-His-Gln-Asp-Ser-Ser-Arg as set forth in Table I from amino acid #15 to #23 is noted to be similar to a tryptic fragment sequence Ser-Thr-Pro-Ala-Gln-Asp-Val-Ser-Arg found in the 28,000 - 30,000 dalton purified bone preparation as described in the "BMP" co-pending applications mentioned above. This fragment set forth in Table I is a portion of the DNA sequence which encodes a bovine BMP-5 protein of the invention. The DNA sequence indicates an open reading frame from the 5' end of the clone of 420 base pairs, encoding a partial peptide of 140 amino acid residues (the first 7 nucleotides are of the adaptors used in the cloning procedure). An in-frame stop codon (TAA) indicates that this clone encodes the carboxy-terminal part of the bovine BMP-5 cartilage/bone protein of the invention.

TABLE I

	TCTAGAGGTGAGAGCAGCCAACAAGAGAAAAAATCAAAACCGCAATAAATCCGGCTCTCAT LeuGluValArgAlaAlaAsnLysArgLysAsnGlnAsnArgAsnLys <u>SerGlySerHis</u> (1) (15)	61
62	CAGGACTCCTCTAGAATGTCCAGTGTGGAGATTATAACACCAGTGAACAAAAACAAGCC <u>GlnAspSerSerArgMetSerSerValGlyAspTyrAsnThrSerGluGlnLysGlnAla</u> (23)	121
122	TGTAAAAAGCATGAACTCTATGTGAGTTTCCGGGATCTGGGATGGCAGGACTGGATTATA CysLysLys <u>HisGluLeuTyrValSerPheArgAspLeuGlyTrpGlnAspTrpIleIle</u> (42)	181
182	GCACCAGAAGGATATGCTGCATTTTATTGTGATGGAGAATGTTCTTTTCCACTCAATGCC AlaProGluGlyTyrAlaAlaPheTyrCysAspGlyGluCysSerPheProLeuAsnAla	241
242	CATATGAATGCCACCAATCATGCCATAGTTCAGACTCTGGTTCACCTGATGTTTCCTGAC <u>HisMetAsnAlaThrAsnHisAlaIleValGlnThrLeuValHisLeuMetPheProAsp</u>	301
302	CACGTACCAAAGCCTTGCTGCGCGACAAACAACTAAATGCCATCTCTGTGTTGTACTTT HisValProLysProCysCysAlaThrAsnLysLeuAsnAlaIleSerValLeuTyrPhe	361
362	GATGACAGCTCCAATGTCATTTTGAAAAAGTACAGAAATATGGTCGTGCGTTCGTGTGGT AspAspSerSerAsnValIleLeuLysLysTyrArgAsnMetValValArgSerCysGly	421
422	TGCCACTAATAGTGCATAATAATGGTAATAAGAAAAAAGATCTGTATGGAGGTTTATGA CysHisEnd (140)	481
481	CTACAATAAAAAATATCTTTCGGATAAAAGGGGAATTTAATAAAATTAGTCTGGCTCATT	540
541	TCATCTCTGTAACCTATGTACAAGAGCATGTATATAGT	578

The remaining positive clones isolated with probes #1, #2, #3, and #4 described above are screened with HEL5 and a further clone is identified that hybridizes under reduced hybridization conditions [5x SSC, 0.1% SDS, 5X Denhardt's, 100 lg/ml salmon sperm DNA standard hybridization buffer (SHB) at 65°C, wash in 2XSSC 0.1% SDS at 65°C]. This clone is plaque purified, a phage plate stock made and bacteriophage DNA isolated. The DNA sequence and derived amino acid sequence of a portion of this clone is shown in Table II. This sequence represents the DNA sequence encoding a BMP-6 cartilage/bone protein.

The first underlined portion of the sequence in Table II from amino acid #97 - amino acid # 105 corresponds to the tryptic fragment found in the 28,000-30,000 dalton purified bovine bone preparation (and its reduced form at approximately 18,000-20,000 dalton reduced form) as described in the "BMP" co-pending applications mentioned above. The second underlined sequence in Table II from amino acid # 124 - amino acid #130 corresponds to the tryptic fragment identified above from which the oligonucleotide probes are designed.

The DNA sequence of Table II indicates an open reading frame of 666 base pairs starting from the 5' end of the sequence of Table II, encoding a partial peptide of 222 amino acid residues. An in-frame stop codon (TGA) indicates that this clone encodes the carboxy-terminal part of a bovine BMP-6 protein of the invention. Based on knowledge of other BMP proteins and other proteins in the TGF- β family, it is predicted that the precursor polypeptide would be cleaved at the three basic residues (ArgArgArg) to yield a mature peptide beginning with residue 90 or 91 of the sequence of Table II.

TABLE II

CTG	CTG	9	GGC	ACG	CGT	18	GCT	GTG	TGG	27	GCC	TCA	GAG	36	GCG	GGC	TGG	45	CTG	GAG	TTT	54	GAC
Leu	Leu	Gly	Thr	Arg	Ala	Val	Trp	Ala	Ser	Glu	Ala	Gly	Trp	Leu	Glu	Phe	Asp						
(1)																							
ATC	ACG	63	GCC	ACC	AGC	72	AAC	CTG	TGG	81	GTC	CTG	ACT	90	CCG	CAG	CAC	99	AAC	ATG	GGG	108	CTG
Ile	Thr	Ala	Thr	Ser	Asn	Leu	Trp	Val	Leu	Thr	Pro	Gln	His	Asn	MET	Gly	Leu						
CAG	CTG	117	AGC	GTG	GTC	126	ACG	CGT	GAT	135	GGG	CTC	AGC	144	ATC	AGC	CCT	153	GGG	GCC	GCG	162	GGC
Gln	Leu	Ser	Val	Val	Thr	Arg	Asp	Gly	Leu	Ser	Ile	Ser	Pro	Gly	Ala	Ala	Gly						
CTG	GTG	171	GGC	AGG	GAC	180	GGC	CCC	TAC	189	GAC	AAG	CAG	198	CCC	TTC	ATG	207	GTG	GCC	TTC	216	TTC
Leu	Val	Gly	Arg	Asp	Gly	Pro	Tyr	Asp	Lys	Gln	Pro	Phe	MET	Val	Ala	Phe	Phe						
AAG	GCC	225	AGT	GAG	GTC	234	CAC	GTG	CGC	243	AGT	GCC	CGG	252	TCG	GCC	CCC	261	GGG	CGG	CGC	270	CGG
Lys	Ala	Ser	Glu	Val	His	Val	Arg	Ser	Ala	Arg	Ser	Ala	Pro	Gly	Arg	Arg	Arg						
CAG	CAG	279	GCC	CGG	AAC	288	CGC	TCC	ACC	297	CCG	GCC	CAG	306	GAC	GTG	TCG	315	CGG	GCC	TCC	324	AGC
Gln	Gln	Ala	Arg	Asn	Arg	Ser	Thr	Pro	Ala	Gln	Asp	Val	Ser	Arg	Ala	Ser	Ser						
GCC	TCA	333	GAC	TAC	AAC	342	AGC	AGC	GAG	351	CTG	AAG	ACG	360	GCC	TGC	CGG	369	AAG	CAT	GAG	378	CTC
Ala	Ser	Asp	Tyr	Asn	Ser	Ser	Ser	Glu	Leu	Lys	Thr	Ala	Cys	Arg	Lys	His	Glu	Leu					
TAC	GTG	387	AGC	TTC	CAG	396	GAC	CTG	GGG	405	TGG	CAG	GAC	414	TGG	ATC	ATT	423	GCC	CCC	AAG	432	GGC
<u>Tyr</u>	<u>Val</u>	<u>Ser</u>	<u>Phe</u>	<u>Gln</u>	<u>Asp</u>	<u>Leu</u>	<u>Gly</u>	<u>Trp</u>	<u>Gln</u>	<u>Asp</u>	<u>Trp</u>	<u>Ile</u>	<u>Ile</u>	<u>Ala</u>	<u>Pro</u>	<u>Lys</u>	<u>Gly</u>						
TAC	GCT	441	GCC	AAC	TAC	450	TGT	GAC	GGA	459	GAA	TGT	TCG	468	TTC	CCT	CTC	477	AAC	GCA	CAC	486	ATG
Tyr	Ala	Ala	Asn	Tyr	Cys	Asp	Gly	Glu	Cys	Ser	Phe	Pro	Leu	Asn	Ala	His	MET						
AAC	GCT	495	ACC	AAC	CAT	504	GCC	ATC	GTG	513	CAG	ACC	CTG	522	GTT	CAC	CTC	531	ATG	AAC	CCC	540	GAG
Asn	Ala	Thr	Asn	His	Ala	Ile	Val	Gln	Thr	Leu	Val	His	Leu	MET	Asn	Pro	Glu						

TABLE II
(cont.)

		549			558		567		576		585		594				
TAC	GTC	CCC	AAA	CCG	TGC	TGC	GCG	CCC	ACG	AAA	CTG	AAC	GCC	ATC	TCG	GTG	CTC
Tyr	Val	Pro	Lys	Pro	Cys	Cys	Ala	Pro	Thr	Lys	Leu	Asn	Ala	Ile	Ser	Val	Leu
		603			612		621		630		639		648				
TAC	TTC	GAC	GAC	AAC	TCC	AAT	GTC	ATC	CTG	AAG	AAG	TAC	CGG	AAC	ATG	GTC	GTA
Tyr	Phe	Asp	Asp	Asn	Ser	Asn	Val	Ile	Leu	Lys	Lys	Tyr	Arg	Asn	MET	Val	Val
		657			666		676		686		696		706				
CGA	GCG	TGT	GGG	TGC	CAC	TGACTCGGGG	TGAGTGGCTG	GGGACGCTGT	GCACACACTG								
Arg	Ala	Cys	Gly	Cys	His												
					(222)												
		716			726		736		746		756		766				
CCTGGACTCC	TGGATCACGT	CCGCCTTAAG	CCCACAGAGG	CCCCCGGGAC	ACAGGAGGAG												
		776			786		796		806		816		826				
ACCCCGAGGC	CACCTTCGGC	TGGCGTTGGC	CTTTCCGCCC	AACGCAGACC	CGAAGGGACC												
		836			846		856		866		876		886				
CTGTCCGCCC	CTTGCTCACA	CCGTGAGCGT	TGTGAGTAGC	CATCGGGCTC	TAGGAAGCAG												

CACTCGAG

EXAMPLE V

Human BMP-5 Proteins

Human cell lines which synthesize BMP-5 and/or BMP-6 mRNAs are identified in the following manner. RNA is isolated from a variety of human cell lines, selected for poly(A)-containing RNA by chromatography on oligo(dT) cellulose, electrophoresed on a formaldehyde-agarose gel, and transferred to nitrocellulose. A nitrocellulose replica of the gel is hybridized to a single stranded M13 ³²P-labeled probe corresponding to the above mentioned BMP-5 EcoRI-BglIII fragment containing nucleotides 1-465 of the sequence of Table I. A strongly hybridizing band is detected in the lane corresponding to the human osteosarcoma cell line U-2OS RNA. Another nitrocellulose replica is hybridized to a single stranded M13 ³²P-labeled probe containing the PstI-SmaI fragment of bovine BMP-6 (corresponding to nucleotides 106-261 of Table II). It is found that several RNA species in the lane corresponding to U-2OS RNA hybridize to this probe.

A cDNA library is made in the vector lambda ZAP (Stratagene) from U-2OS poly(A)-containing RNA using established techniques (Toole et al.). 750,000 recombinants of this library are plated and duplicate nitrocellulose replicas made. The SmaI fragment of bovine BMP-6 corresponding to nucleotides 259-751 of Table II is labeled by nick-translation and hybridized to both sets of filters in SHB at 65°. One set of filters is washed under stringent conditions (0.2X SSC, 0.1% SDS at 65°), the other under reduced stringency conditions (1X SSC, 0.1% SDS at 65°). Many duplicate hybridizing recombinants (approximately 162) are noted. 24 are picked and replated for secondaries. Three nitrocellulose replicas are made of each plate. One is hybridized to the BMP-6 SmaI probe, one to a nick-translated BMP-6 PstI-SacI fragment (nucleotides 106-378 of Table II), and the third to the nick-translated BMP-5 XbaI fragments (nucleotides 1-76 of Table I). Hybridization and washes are carried out under stringent conditions.

17 clones that hybridized to the third probe more strongly than to the second probe are plaque purified. DNA sequence

analysis of one of these, U2-16, indicated that it encodes human BMP-5. U2-16 was deposited with the American Type Culture Collection (ATCC), Rockville, Maryland on June 22, 1989 under accession number ATCC 68109. U2-16 contains an insert of approximately 2.1 Kb. The DNA sequence and derived amino acid sequence of U2-16 is shown below in Table III. This clone is expected to contain all of the nucleotide sequence necessary to encode the BMP-5 proteins. The cDNA sequence of Table III contains an open reading frame of 1362 bp, encoding a protein of 454 amino acids, preceded by a 5' untranslated region of 700 bp with stop codons in all frames, and contains a 3' untranslated region of 90 bp following the in frame stop codon (TAA).

This protein of 454 amino acids has a molecular weight of approximately 52,000 kd as predicted by its amino acid sequence, and is contemplated to represent the primary translation product. Based on knowledge of other BMP proteins and other proteins within the TGF- β family, cleavage of the precursor polypeptide may occur after the tribasic peptide Lys Arg Lys yielding a 132 amino acid mature peptide beginning with amino acid # 323 "Asn". However, the presence of di- or tribasic amino acid sequence is not an absolute requirement for proteolytic processing, as a number of prohormones are known to be processed after single arginines which conform to a consensus cleavage sequence arginine-X-X-arginine. It is therefore contemplated that the precursor polypeptide is proteolytically processed after the Arg-Ser-Val-Arg sequence yielding a polypeptide comprising 138 amino acids from amino acid #317 (Ala) to #454 (His) as shown in Table III with a calculated molecular weight of 15.6 kD. The processing of BMP-5 into the mature form is expected to involve dimerization and removal of the N-terminal region in a manner analogous to the processing of the related protein TGF- β [L.E. Gentry, et al., Molec. & Cell. Biol. 8:4162 (1988); R. Dernyck, et al., Nature 316:701 (1985)].

It is contemplated therefore that the mature active species of BMP-5 comprises a homodimer of 2 polypeptide subunits each subunit comprising amino acid #323 - #454 with a predicted molecular weight

of approximately 15,000 daltons. Further active BMP-5 species are contemplated, for example, proprotein dimers or proprotein subunits linked to mature subunits. Additional active species may comprise amino acid #329 - #454 such species including homologous the tryptic sequences found in the purified bovine material. Also contemplated are BMP-5 proteins comprising amino acids #353 - #454 thereby including the first conserved cysteine residue

The underlined sequence of Table III from amino acid #329 to #337 Ser-Ser-Ser-His-Gln-Asp-Ser-Ser-Arg shares homology with the bovine sequence of Table I from amino acid #15 to #23 as discussed above in Example IV. Each of these sequences shares homology with a tryptic fragment sequence Ser-Thr-Pro-Ala-Gln-Asp-Val-Ser-Arg found in the 28,000 - 30,000 dalton purified bone preparation (and its reduced form at approximately 18,000 - 20,000 daltons) as described in the "BMP" co-pending applications mentioned above.

The underlined sequence of Table III from amino acid #356 to #362 His-Glu-Leu-Tyr-Val-Ser-Phe corresponds to the tryptic fragment identified in the bovine bone preparation described above from which the oligonucleotide probes are designed.

TABLE III

Human

10	20	30	40	50
CTGGTATATT	TGTGCCTGCT	GGAGGTGGAA	TTAACAGTAA	GAAGGAGAAA
60	70	80	90	100
GGGATTGAAT	GGA CT TACAG	GAAGGATTTC	AAGTAAATTC	AGGGAAACAC
110	120	130	140	150
ATTTACTTGA	ATAGTACAAC	CTAGAGTATT	ATTTTACACT	AAGACGACAC
160	170	180	190	200
AAAAGATGTT	AAAGTTATCA	CCAAGCTGCC	GGACAGATAT	ATATTCCAAC
210	220	230	240	250
ACCAAGGTGC	AGATCAGCAT	AGATCTGTGA	TTCAGAAATC	AGGATTTGTT
260	270	280	290	300
TTGGAAAGAG	CTCAAGGCTT	GAGAAGAACT	CAAAAGCAAG	TGAAGATTAC
310	320	330	340	350
TTTGGGA ACT	ACAGTTTATC	AGAAGATCAA	CTTTTGCTAA	TTCAAATACC
360	370	380	390	400
AAAGGCCTGA	TTATCATAAA	TTCATATAGG	AATGCATAGG	TCATCTGATC
410	420	430	440	450
AAATAATATT	AGCCGTCTTC	TGCTACATCA	ATGCAGCAAA	AACTCTTAAC
460	470	480	490	500
AACTGTGGAT	AATTGGAAAT	CTGAGTTTCA	GCTTTCTTAG	AAATAACTAC
510	520	530	540	550
TCTTGACATA	TTCCAAAATA	TTTAAAATAG	GACAGGAAAA	TCGGTGAGGA
560	570	580	590	600
TGTTGTGCTC	AGAAATGTCA	CTGTCATGAA	AAATAGGTAA	ATTTGTTTTT
610	620	630	640	650
TCAGCTACTG	GGAAACTGTA	CCTCCTAGAA	CCTTAGGTTT	TTTTTTTTTT
660	670	680	690	700
AAGAGGACAA	GAAGGACTAA	AAATATCAAC	TTTTGCTTTT	GGACAAAA

TABLE III(a)

701	710	719	728	737
ATG CAT CTG ACT GTA TTT TTA CTT AAG GGT ATT GTG GGT TTC CTC				
MET His Leu Thr Val Phe Leu Leu Lys Gly Ile Val Gly Phe Leu				
(1)				
746	755	764	773	782
TGG AGC TGC TGG GTT CTA GTG GGT TAT GCA AAA GGA GGT TTG GGA				
Trp Ser Cys Trp Val Leu Val Gly Tyr Ala Lys Gly Gly Leu Gly				
791	800	809	818	827
GAC AAT CAT GTT CAC TCC AGT TTT ATT TAT AGA AGA CTA CGG AAC				
Asp Asn His Val His Ser Ser Phe Ile Tyr Arg Arg Leu Arg Asn				
836	845	854	863	872
CAC GAA AGA CGG GAA ATA CAA AGG GAA ATT CTC TCT ATC TTG GGT				
His Glu Arg Arg Glu Ile Gln Arg Glu Ile Leu Ser Ile Leu Gly				
881	890	899	908	917
TTG CCT CAC AGA CCC AGA CCA TTT TCA CCT GGA AAA ATG ACC AAT				
Leu Pro His Arg Pro Arg Pro Phe Ser Pro Gly Lys Gln Ala Ser				
926	935	944	953	962
CAA GCG TCC TCT GCA CCT CTC TTT ATG CTG GAT CTC TAC AAT GCC				
Ser Ala Pro Leu Phe MET Leu Asp Leu Tyr Asn Ala MET Thr Asn				
971	980	989	998	1007
GAA GAA AAT CCT GAA GAG TCG GAG TAC TCA GTA AGG GCA TCC TTG				
Glu Glu Asn Pro Glu Glu Ser Glu Tyr Ser Val Arg Ala Ser Leu				
1016	1025	1034	1043	1052
GCA GAA GAG ACC AGA GGG GCA AGA AAG GGA TAC CCA GCC TCT CCC				
Ala Glu Glu Thr Arg Gly Ala Arg Lys Gly Tyr Pro Ala Ser Pro				
1061	1070	1079	1088	1097
AAT GGG TAT CCT CGT CGC ATA CAG TTA TCT CGG ACG ACT CCT CTG				
Asn Gly Tyr Pro Arg Arg Ile Gln Leu Ser Arg Thr Thr Pro Leu				
1106	1115	1124	1133	1142
ACC ACC CAG AGT CCT CCT CTA GCC AGC CTC CAT GAT ACC AAC TTT				
Thr Thr Gln Ser Pro Pro Leu Ala Ser Leu His Asp Thr Asn Phe				
1151	1160	1169	1178	1187
CTG AAT GAT GCT GAC ATG GTC ATG AGC TTT GTC AAC TTA GTT GAA				
Leu Asn Asp Ala Asp MET Val MET Ser Phe Val Asn Leu Val Glu				
1196	1205	1214	1223	1232
AGA GAC AAG GAT TTT TCT CAC CAG CGA AGG CAT TAC AAA GAA TTT				
Arg Asp Lys Asp Phe Ser His Gln Arg Arg His Tyr Lys Glu Phe				

TABLE III(b)

1241	1250	1259	1268	1277
CGA TTT GAT CTT ACC CAA ATT CCT CAT GGA GAG GCA GTG ACA GCA				
Arg Phe Asp Leu Thr Gln Ile Pro His Gly Glu Ala Val Thr Ala				
1286	1295	1304	1313	1322
GCT GAA TTC CGG ATA TAC AAG GAC CGG AGC AAC AAC CGA TTT GAA				
Ala Glu Phe Arg Ile Tyr Lys Asp Arg Ser Asn Asn Arg Phe Glu				
1331	1340	1349	1358	1367
AAT GAA ACA ATT AAG ATT AGC ATA TAT CAA ATC ATC AAG GAA TAC				
Asn Glu Thr Ile Lys Ile Ser Ile Tyr Gln Ile Ile Lys Glu Tyr				
1376	1385	1394	1403	1412
ACA AAT AGG GAT GCA GAT CTG TTC TTG TTA GAC ACA AGA AAG GCC				
Thr Asn Arg Asp Ala Asp Leu Phe Leu Leu Asp Thr Arg Lys Ala				
1421	1430	1439	1448	1457
CAA GCT TTA GAT GTG GGT TGG CTT GTC TTT GAT ATC ACT GTG ACC				
Gln Ala Leu Asp Val Gly Trp Leu Val Phe Asp Ile Thr Val Thr				
1466	1475	1484	1493	1502
AGC AAT CAT TGG GTG ATT AAT CCC CAG AAT AAT TTG GGC TTA CAG				
Ser Asn His Trp Val Ile Asn Pro Gln Asn Asn Leu Gly Leu Gln				
1511	1520	1529	1538	1547
CTC TGT GCA GAA ACA GGG GAT GGA CGC AGT ATC AAC GTA AAA TCT				
Leu Cys Ala Glu Thr Gly Asp Gly Arg Ser Ile Asn Val Lys Ser				
1556	1565	1574	1583	1592
GCT GGT CTT GTG GGA AGA CAG GGA CCT CAG TCA AAA CAA CCA TTC				
Ala Gly Leu Val Gly Arg Gln Gly Pro Gln Ser Lys Gln Pro Phe				
1601	1610	1619	1628	1637
ATG GTG GCC TTC TTC AAG GCG AGT GAG GTA CTT CTT CGA TCC GTG				
MET Val Ala Phe Phe Lys Ala Ser Glu Val Leu Leu Arg Ser Val				
1646	1655	1664	1673	1682
AGA GCA GCC AAC AAA CGA AAA AAT CAA AAC CGC AAT AAA TCC AGC				
Arg Ala Ala Asn Lys Arg Lys Asn Gln Asn Arg Asn Lys <u>Ser Ser</u>				
				(329)
1691	1700	1709	1718	1727
TCT CAT CAG GAC TCC TCC AGA ATG TCC AGT GTT GGA GAT TAT AAC				
<u>Ser His Gln Asp Ser Ser Arg</u> MET Ser Ser Val Gly Asp Tyr Asn				
				(337)

TABLE III(c)

1736	1745	1754	1763	1772	
ACA AGT GAG CAA AAA CAA GCC TGT AAG AAG CAC GAA CTC TAT GTG					
Thr Ser Glu Gln Lys Gln Ala Cys Lys Lys <u>His Glu Leu Tyr Val</u>					
			(356)		
1781	1790	1799	1808	1817	
AGC TTC CGG GAT CTG GGA TGG CAG GAC TGG ATT ATA GCA CCA GAA					
<u>Ser Phe</u> Arg Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu					
(362)					
1826	1835	1844	1853	1862	
GGA TAC GCT GCA TTT TAT TGT GAT GGA GAA TGT TCT TTT CCA CTT					
Gly Tyr Ala Ala Phe Tyr Cys Asp Gly Glu Cys Ser Phe Pro Leu					
1871	1880	1889	1898	1907	
AAC GCC CAT ATG AAT GCC ACC AAC CAC GCT ATA GTT CAG ACT CTG					
Asn Ala His MET Asn Ala Thr Asn His Ala Ile Val Gln Thr Leu					
1916	1925	1934	1943	1952	
GTT CAT CTG ATG TTT CCT GAC CAC GTA CCA AAG CCT TGT TGT GCT					
Val His Leu MET Phe Pro Asp His Val Pro Lys Pro Cys Cys Ala					
1961	1970	1979	1988	1997	
CCA ACC AAA TTA AAT GCC ATC TCT GTT CTG TAC TTT GAT GAC AGC					
Pro Thr Lys Leu Asn Ala Ile Ser Val Leu Tyr Phe Asp Asp Ser					
2006	2015	2024	2033	2042	
TCC AAT GTC ATT TTG AAA AAA TAT AGA AAT ATG GTA GTA CGC TCA					
Ser Asn Val Ile Leu Lys Lys Tyr Arg Asn MET Val Val Arg Ser					
			(450)		
2051	2060	2070	2080	2090	2100
TGT GGC TGC CAC TAATATTAAA TAATATTGAT AATAACAAAA AGATCTGTAT					
Cys Gly Cys His					
2110	2120	2130	2140	2150	
TAAGGTTTAT GGCTGCAATA AAAAGCATAC TTTCAGACAA ACAGAAAAAA AAA					

The invention encompasses the corresponding bovine and human BMP-5 genomic sequences. These genes can be isolated using the cDNA sequences set forth in Table I and Table III as probes to screen genomic libraries using techniques known to those skilled in the art.

When the tryptic sequence His-Glu-Leu-Tyr-Val-Ser-Phe-(Ser) described above was identified, it was noted to be similar to the sequence His-Pro-Leu-Tyr-Val-Asp-Phe-Ser found in the bovine and human cartilage/bone protein BMP-2A sequence, for instance as described in co-pending U.S. application Serial No. 179,100. Human BMP-5 shares homology with other BMP molecules as well as other members of the TGF- β superfamily of molecules. The cysteine-rich carboxy-terminal 102 amino acids residues of human BMP-5 shares the following homologies with BMP proteins disclosed in copending applications described above: 61% identity with BMP-2; 43% identity with BMP-3, 59% identity with BMP-4; 91% identity with BMP-6; and 88% identity with BMP-7. Human BMP-5 further shares the following homologies: 38% identity with TGF- β 3; 37% identity with TGF- β 2; 36% identity with TGF- β 1; 25% identity with Mullerian Inhibiting Substance (MIS), a testicular glycoprotein that causes regression of the Mullerian duct during development of the male embryo; 25% identity with inhibin a; 38% identity with inhibin b_B; 45% identity with inhibin b_A; 56% identity with Vg1, a Xenopus factor which may be involved in mesoderm induction in early embryogenesis (Lyons, et al., PNAS USA 86:4554-4558 (1989)); and 57% identity with Dpp the product of the Drosophila decapentaplegic locus which is required for dorsal-ventral specification in early embryogenesis and is involved in various other developmental processes at later stages of development [Padgett, et al., Nature 325:81-84 (1987)].

The procedures described above and additional methods known to those skilled in the art may be employed to isolate other related proteins of interest by utilizing the bovine or

human proteins as a probe source. Such other proteins may find similar utility in, inter alia, fracture repair, wound healing and tissue repair.

5 **EXAMPLE VI**

Expression of the BMP-5 Proteins

10 In order to produce bovine, human or other mammalian proteins of the invention, the DNA encoding it is transferred into an appropriate expression vector and introduced into mammalian cells or other preferred eukaryotic or prokaryotic hosts by conventional genetic engineering techniques. It is contemplated that the preferred expression system for biologically active recombinant human proteins of the invention may be stably transformed mammalian cells. For transient
15 expression, the cell line of choice is SV40 transformed African green monkey kidney COS-1 or COS-7 which typically produce moderate amounts of the protein encoded within the plasmid for a period of 1-4 days. For stable high level expression, it is further contemplated that the preferred
20 mammalian cells will be Chinese hamster ovary (CHO) cells.

 The transformed host cells are cultured and the BMP-5 protein expressed thereby is recovered, isolated and purified. Characterization of expressed proteins is carried out using standard techniques. For example, characterization may include
25 pulse labeling with [³⁵S] methionine or cysteine and analysis by polyacrylamide electrophoresis. The recombinantly expressed BMP-5 proteins are free of proteinaceous materials with which they are co-produced and with which they ordinarily are associated in nature, as well as from other contaminants, such
30 as materials found in the culture media.

 In order to express biologically active human BMP-5 a selected host cell is transformed, using techniques known to those skilled in the art of genetic engineering, with a DNA sequence encoding human BMP-5 protein. The DNA comprises the
35 nucleotide sequence from nucleotide #1665 to #2060 set forth in

Table III encoding amino acid #323 to #454. The DNA may comprise the DNA sequence from nucleotide # 699 to # 2060 set forth in Table III. The transformed host cells are cultured and the BMP-5 protein comprising the amino acid sequence from amino acid # 323 to amino acid # 454 set forth in Table III is expressed. The expressed protein is recovered, isolated and purified from the culture and culture medium. The purified protein is substantially free from other proteinaceous materials with which it is co-produced, and from other contaminants.

A. Vector Construction

As described above, numerous expression vectors known in the art may be utilized in the expression of BMP proteins of the invention. The vector utilized in the following examples are pMT21, a derivative of pMT₂, and pEMC2 β 1 derived from pMT21 though other vectors may be suitable in practice of the invention.

pMT₂ is derived from pMT2-VWF, which has been deposited with the American Type Culture Collection (ATCC), Rockville, MD (USA) under accession number ATCC 67122 under the provisions of the Budapest Treaty. EcoRI digestion excises the cDNA insert present in pMT-VWF, yielding pMT2 in linear form which can be ligated and used to transform E. Coli HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional methods.

pMT21 is then constructed using loopout/in mutagenesis [Morinaga, et al., Biotechnology 84:636 (1984)]. This removes bases 1075 to 1170 (inclusive). In addition it inserts the following sequence: 5' TCGA 3'. This sequence completes a new restriction site, XhoI. This plasmid now contains 3 unique cloning sites PstI, EcoRI, and XhoI.

In addition, pMT21 is digested with EcoRV and XhoI, treating the digested DNA with Klenow fragment of DNA polymerase I and ligating ClaI linkers (NEBio Labs, CATCGATG).

This removes bases 2171 to 2420 starting from the HindIII site near the SV40 origin of replication and enhancer sequences of pMT2 and introduces a unique Cla I site, but leaves the adenovirus VAI gene intact.

5 pEMC2 β 1 is derived from pMT21. pMT21 is cut with EcoRI and XhoI which cleaves the plasmid two adjacent cloning sites situated after the IgG intron. An EMCV fragment of 508 base pairs is cut from pMT₂ECAT₁ [S.K. Jong, et al., J. Virol. 63:1651-1660 (1989)] with the restriction enzymes EcoRI and
10 TagoI. A pair of oligonucleotides 68 nucleotides cga gggttaaaaaa cgtctaggcc ccccgaacca cggggacgtg gttttccttt gaaaaacacg attgc in length are synthesized to duplicate the EMCV sequence up to the ATG. The ATG is changed to an ATT, and a C is added, creating a XhoI site at the 3' end. A tag~~x~~I site
15 is situated at the 5' end. Ligation of the MT21 EcoRI to XhoI fragment to the EMCV EcoRI to Tag~~x~~I fragment and to the Tag~~x~~I/XhoI oligonucleotides produces the vector EMC β B1. This vector contains the SV40 origin of replication and enhancer, the adenovirus major late promoter, a cDNA copy of the majority
20 of the adenovirus tripartite leader sequence, a small hybrid intervening sequence, an SV40 polyadenylation signal and the adenovirus VA I gene, DHFR and B-lactamase markers and an EMC sequence, in appropriate relationships to direct the high level expression of the desired cDNA in mammalian cells.

25 B. BMP-5 Vector Construction

A derivative of the BMP-5 cDNA sequence set forth in Table III comprising the the nucleotide sequence from nucleotide #699 to #2070 is specifically amplified. The oligonucleotides
30 CGACCTGCAGCCACCATGCATCTGACTGTA and TGCCTGCAGTTTAAATATTAGTGGCAGC are utilized as primers to allow the amplification of nucleotide sequence #699 to #2070 of Table III from the insert of clone U2-16 described above in Example V. This procedure introduces the nucleotide sequence CGACCTGCAGCCACC immediately
35 preceeding nucleotide #699 and the nucleotide sequence

CTGCAGGCA immediately following nucleotide #2070. The addition of these sequences results in the creation of PstI restriction endonuclease recognition sites at both ends of the amplified DNA fragment. The resulting amplified DNA product of this procedure is digested with the restriction endonuclease PstI and subcloned into the PstI site of the pMT2 derivative pMT21 described above. The resulting clone is designated H5/5/pMT.

The insert of H5/5/pMT is excised by PstI digestion and subcloned into the plasmid vector pSP65 at the PstI site resulting in BMP5/SP6. BMP5/SP6 and U2-16 are digested with the restriction endonucleases NsiI and NdeI to excise the portion of their inserts corresponding to nucleotides #704 to #1876 of Table III. The resulting 1173 nucleotide NsiI-NdeI fragment of clone U2-16 is ligated into the NsiI-NdeI site of BMP5/SP6 from which the corresponding 1173 nucleotide NsiI-NdeI fragment had been removed. The resulting clone is designated BMP5mix/SP64.

Direct DNA sequence analysis of BMP5mix/SP64 is performed to confirm identity of the nucleotide sequences produced by the amplification to those set forth in Table III. The clone BMP5mix/SP64 is digested with the restriction endonuclease PstI resulting in the excision of an insert comprising the nucleotides #699 to #2070 of Table III and the additional sequences containing the PstI recognition sites as described above. The resulting 1382 nucleotide PstI fragment is subcloned into the PstI site of the pMT2 derivative pMT21 and pEMC2 β 1. These clones are designated BMP5mix/pMT21#2 and Bmp5mix/EMC#11.

Example VII

Transient COS Cell Expression

To obtain transient expression of BMP-5 proteins a vector containing the cDNA for BMP-5, BMP5mix/pMT21#2, is transfected into COS-1 cells using the electroporation method. Other

suitable transfection methods include DEAE-dextran, and lipofection. Approximately 48 hours later, cells are analysed for expression of both intracellular and secreted BMP-5 protein by metabolic labelling with [³⁵S] methionine and polyacrylamide gel electrophoresis. Intracellular BMP is analyzed in cells which are treated with tunicamycin, an inhibitor of N-linked glycosylation. In tunicamycin-treated cells, the nonglycosylated primary translation product migrates as a homogeneous band of predictable size and is often easier to discern in polyacrylamide gels than the glycosylated form of the protein. In each case, intracellular protein in tunicamycin-treated cells is compared to a duplicate plate of transfected, but untreated COS-1 cells.

The results demonstrate that intracellular forms of BMP-5 of approximately 52 Kd and 57 Kd are made by COS cells. The 52 Kd protein is the size predicted by the primary sequence of the the BMP-5 cDNA clone. Following treatment of the cells with tunicamycin, only the 52 Kd form of BMP-5 is made, suggesting that the 57 Kd protein is a glycosylated derivative of the 52 Kd primary translation product. The 57 Kd protein is secreted into the conditioned medium and is apparently not efficiently processed by COS-1 cells into the pro and mature peptides.

Example VIII

CHO Cell Expression

DHFR deficient CHO cells (DUKX B11) are transfected by electroporation with BMP-5 expression vectors described above, and selected for expression of DHFR by growth in nucleoside-free media. Other methods of transfection, including but not limited to CaPO_4 precipitation, protoplast fusion, microinjection, and lipofection, may also be employed. In order to obtain higher levels of expression more expediently, cells may be selected in nucleoside-free media supplemented with 5 nM, 20 nM or 100 nM MTX. Since the DHFR selectable marker is physically linked to the BMP-5 cDNA as the second

gene of a bicistronic coding region, cells which express DHFR should also express the BMP-5 encoded within the upstream cistron. Either single clones, or pools of combined clones, are expanded and analyzed for expression of BMP protein. Cells are selected in stepwise increasing concentrations of MTX (5 nM, 20 nM, 100 nM, 500 nM, 2 uM, 10 uM, and 100 uM) in order to obtain cell lines which contain multiple copies of the expression vector DNA by virtue of gene amplification, and hence secrete large amounts of BMP-5 protein.

Using standard techniques cell lines are screened for expression of BMP-5 RNA, protein or activity, and high expressing cell lines are cloned or recloned at the appropriate level of selection to obtain a more homogeneous population of cells. The resultant cell line is then further characterized for BMP-5 DNA sequences, and expression of BMP-5 RNA and protein. Suitable cell lines can then be used for producing recombinant BMP protein.

The BMP-5 vector BMP5mix/pMT21#2 and BMP5mix/EMC#11 described above are transfected into CHO cells by electroporation, and cells are selected for expression of DHFR in nucleoside free medium. Clonal cell lines are obtained from individual colonies and are subsequently selected stepwise for resistance to MTX, and are analyzed for secretion of BMP-5 proteins. In some cases cell lines may be maintained as pools and cloned at later stages of MTX selection. One particular cell line further described, is designated 5E10 is sequentially selected for resistance to .02uM, 0.1mM, 0.5uM and 2.0uM MTX to obtain amplified expression of BMP-5.

The amount of BMP-5 recovered in conditioned medium from 5E10 and other cell lines that express BMP-5 can be increased by including heparin, suramin, dextran sulfate, pectic acid, sodium sulfate, or related compounds in the growth medium.

As described in Example V. the cDNA for BMP-5 encodes a protein of approximately 52 kD. Following processing within the cell that includes, but may not be limited to, propeptide

cleavage, glycosylation, and dimer or multimer formation, multiple BMP-5 peptides are produced. There are at least 4 candidate peptides for processed forms of the BMP-5 protein discernable following SDS PAGE under reducing conditions; a peptide of approximately 65kD, a peptide of approximately 35kD, and a doublet of approximately 22 kD molecular weight. Other less abundant BMP-5 peptides may also be present. By comparison to the processing of other related BMP molecules and the related protein TGF-beta, the 65 Kd protein likely represents unprocessed BMP-5, the 35 Kd species represents the propeptide, and the 22 Kd doublet represents the mature peptide.

Material from a BMP-5 cell line is analyzed in a 2-dimensional gel system. In the first dimension, proteins are electrophoresed under nonreducing conditions. The material is then reduced, and electrophoresed in a second polyacrylamide gel. Proteins that form disulfide-bonded dimers or multimers will run below a diagonal across the second reduced gel. Results from analysis of BMP-5 protein indicates that a significant amount of the mature BMP-5 peptides can form homodimers of approximately 30-35 kD that reduce to the 22 kD doublet observed in one dimensional reduced gels. A fraction of the mature peptides are apparently in a disulfide-bonded complex with the pro peptide. The amount of this complex is minor relative to the mature homodimer. In addition, some of the unprocessed protein can apparently form homodimers or homomultimers.

EXAMPLE IX

Purification and Biological Activity of Expressed BMP-5 Proteins

To measure the biological activity of the expressed BMP-5 proteins obtained in Example VIII above, the BMP-5 proteins are recovered from the culture media and purified by isolating them from other proteinaceous materials with which they are co-

produced, as well as from other contaminants. BMP-5 proteins may be partially purified on a Heparin Sepharose column and further purified using standard purification techniques known to those skilled in the art. The BMP-5 protein is mixed with 20 mg of rat matrix and then assayed for in vivo bone and/or cartilage formation activity by the Rosen-modified Sampath - Reddi assay. A mock transfection supernatant fractionation is used as a control.

The implants containing rat matrix to which specific amounts of human BMP-5 proteins of the invention have been added are removed from rats after approximately seven days and processed for histological evaluation. Representative sections from each implant are stained for the presence of new bone mineral with von Kossa and acid fuchsin, and for the presence of cartilage-specific matrix formation using toluidine blue. The types of cells present within the section, as well as the extent to which these cells display phenotype are evaluated and scored as described in Example III.

A. Purification of BMP-5 Proteins

(1) As one example of BMP-5 purification 4 ml of the collected post transfection conditioned medium supernatant from one 100 mm culture dish is concentrated approximately 10 fold by ultrafiltration on a YM 10 membrane and then dialyzed against 20mM Tris, 0.15 M NaCl, pH 7.4 (starting buffer). This material is then applied to a 1.1 ml Heparin Sepharose column in starting buffer. Unbound proteins are removed by an 8 ml wash of starting buffer, and bound proteins, including proteins of the invention, are desorbed by a 3-4 ml wash of 20 mM Tris, 2.0 M NaCl, pH 7.4.

The proteins bound by the Heparin column are concentrated approximately 10-fold on a Centricon 10 and the salt reduced by diafiltration with 0.1% trifluoroacetic acid.

Further purification may be achieved by preparative NaDodSO₄/PAGE [Laemmli, Nature 227:680-685 (1970)]. For

instance, approximately 300 μ g of protein is applied to a 1.5-mm-thick 12.5% gel: recovery is estimated by adding L-[³⁵S]methionine-labeled BMP protein purified over heparin-Sepharose as described above. Protein may be visualized by copper staining of an adjacent lane [Lee, et al., Anal. Biochem. 166:308-312 (1987)]. Appropriate bands are excised and extracted in 0.1% NaDodSO₄/20 mM Tris, pH 8.0. The supernatant may be acidified with 10% CF₃COOH to pH 3 and the proteins are desalted on 5.0 x 0.46 cm Vydac C₄ column (The Separations Group, Hesperia, CA) developed with a gradient of 0.1% CF₃COOH to 90% acetonitrile/0.1% CFF₃COOH.

(2) In another example, soluble heparin (100ug/ml) is removed from of BMP-5 protein in conditioned media from 5E10(2) 2.0MTX (described above) using butyl TSK hydrophobic interactive chromatography (HIC). The conditioned media is brought to 2M NaCl by addition of solid NaCl. The conditioned media is then loaded on butyl TSK equilibrated in 2M NaCl, 50mM Tris, pH 7.4 washed with 0 M NaCl, 50mM Tris, Ph 7.4, followed by elution with 1% Np-40, 6M urea, 50mM Tris, pH 7.4 resulting in approximately 98% removal of soluble heparin.

The resulting material is then subjected to heparin sepharose chromatography. The material is directly loaded onto a heparin column equilibrated in 50mM Tris, 6M urea, 0M NaCl, washed and eluted with a gradient of 0-2M NaCl. This material is analyzed by western blot and the BMP-5 containing fractions (0.3 - 0.8 M NaCl) are pooled. The antibody is directed against the C-terminal presumed mature portion. Proteins of 35-40 kD non-reduced, 20-22 kD reduced, and higher molecular weight dimers are observed.

The BMP-5 containing fractions are concentrated and diafiltered to bring the sample to 0.1% TFA loaded onto a reverse phase column and eluted with a gradient from 30% to 60% B (A=.1% TFA; B = .1% TFA in 90% acetonitrile) in 75 min at 1ml/min. SDS-PAGE analysis reveals several molecular weight

species of BMP-5 proteins which are further described below. The mature species which is contemplated to comprise a homodimer of amino acids #317-#454 as shown in Table III comprises approximately 46-49% of the resulting molecular weight species.

B. Characterization of BMP-5 Proteins

One dimensional Western blot analysis reveals several molecular weight species including 98kDa, 72kDa 50kDa and 35-40kDa. Upon reduction the following species are seen 68kDa, 43kDa and 20-22 kDa. The non-reduced 98kDa species is contemplated to comprise a homodimer of two 50kDa subunits each comprising amino acids #28 # 454 as shown in Table III. The 72kDa species is contemplated to comprise a heterodimer of a 50kDa subunit (comprising amino acids #28- #454 of Table III as described above) and a 20kDa subunit comprising amino acids ~~#317 - - #454 as shown in Table III.~~ The 35-40kDa species is contemplated to represent the mature species comprising a homodimer of two 20 kDa subunits each comprising amino acids #317 - #454 as shown in Table III.

C. BMP-5 Activity

BMP-5 (containing 100ug/ml soluble heparin) purified in a preliminary experiment over octyl-sepharose (HIC) [see description below] then over heparin sepharose in a manner similar to the butyl then heparin steps described above is mixed with 20 mg rat matrix and implanted for 10 days according to the rat ectopic assay described above in Example III. Approximately 1-3 ug BMP-5 protein from the heparin sepharose step results in the formation of cartilage and bone. The octyl-sepharose purification step is carried out by adding solid $(\text{NH}_4)_2\text{SO}_4$ to BMP-5 conditioned media containing 100ug/ml soluble heparin to a final concentration of 1M. This is loaded onto a column of octly-sepharose equilabrated in 1M $(\text{NH}_4)_2\text{SO}_4$, 50mM Tris pH 7.4. The column is washed with starting buffer

then with 50mM Tris pH 7.4 and eluted with 50mM Tris, 6M urea, 0.2% octly glucoside pH 7.4. Purification over heparin sepharose is by step gradient, washed with 50mM Tris, 0.15M NaCl, 6M urea pH 7.4, eluted with 50mM Tris, 2M NaCl, 6M urea pH 7.4. The material implanted is 2M NaCl.

The foregoing descriptions detail presently preferred embodiments of the present invention. Numerous modifications and variations in practice thereof are expected to occur to those skilled in the art upon consideration of these descriptions. Those modifications and variations are believed to be encompassed within the claims appended hereto.

What is claimed is:

1. A purified human BMP-5 protein comprising the amino acid sequence from amino acid #323 to amino acid #454 as shown in Table III.
2. A BMP-5 protein of claim 1 comprising the amino acid sequence from amino acid #317 to #454 as shown in Table III.
3. A purified human BMP-5 protein produced by the steps of
 - (a) culturing a cell transformed with a DNA sequence comprising the DNA sequence of Table III from nucleotide # 1665 to # 2060 or a sequence substantially homologous thereto; and
 - (b) recovering from said culture medium a protein comprising the amino acid sequence from amino acid # 323 to amino acid # 454 as shown in Table III or a sequence substantially homologous thereto.
4. A purified human BMP-5 protein produced by the steps of
 - (a) culturing a cell transformed with a DNA sequence comprising the DNA sequence of Table III from nucleotide # 699 to # 2060 or a sequence substantially homologous thereto; and
 - (b) recovering from said culture medium a protein comprising the amino acid sequence from amino acid #323 to amino acid # 454 as shown in Table III or a sequence substantially homologous thereto.
5. A purified BMP-5 protein produced by the steps of
 - (a) culturing a cell transformed with a DNA sequence which hybridizes to the DNA sequence of Table III under stringent hybridization conditions; and
 - (b) recovering from said culture medium a protein

characterized by the ability to induce cartilage and/or bone formation in the Rosen-modified Sampath-Reddi assay.

6. A protein of claim 1 further characterized by the ability to demonstrate cartilage and/or bone formation.
7. A protein of claim 4 further characterized by the ability of 1 μ g of said protein to score at least +2 in the Rosen-modified Sampath-Reddi assay.
8. A DNA sequence encoding a protein of claim 1.
9. A DNA sequence encoding a protein of claim 4.
10. A host cell transformed with a DNA of claim 6.
11. A method for producing a purified BMP-5 protein said method comprising the steps of
 - (a) culturing in a suitable culture medium cells transformed with a DNA sequence comprising the DNA sequence from nucleotide # 699 to # 2060 of Table III; and
 - (b) isolating and purifying said protein from said culture medium.
12. A pharmaceutical composition comprising an effective amount of a protein of claim 1 in admixture with a pharmaceutically acceptable vehicle.
13. A pharmaceutical formulation for bone and/or cartilage formation comprising an effective amount of a protein of claim 1 in a pharmaceutically acceptable vehicle.
14. A composition of claim 12 further comprising a matrix for

supporting said composition and providing a surface for bone and/or cartilage growth.

15. The composition of claim 14 wherein said matrix comprises a material selected from the group consisting of hydroxyapatite, collagen, polylactic acid and tricalcium phosphate.

16. A method for inducing bone and/or cartilage formation in a patient in need of same comprising administering to said patient an effective amount of the composition of claim 13.

17. A pharmaceutical composition for wound healing and tissue repair said composition comprising an effective amount of the protein of claim 1 in a pharmaceutically acceptable vehicle.

18. A method for treating wounds and/or tissue repair in a patient in need of same comprising administering to said patient an effective amount of the composition of claim 12.

19. An isolated DNA sequence encoding a BMP-5 protein said DNA sequence comprising substantially the nucleotide sequence or a portion thereof selected from the group consisting of:

(a) nucleotide # 699 through nucleotide # 2060 of Table III; and

(b) sequences which

(1) hybridize to sequence (a) under stringent hybridization conditions; and

(2) encode a protein characterized by the ability of 1 μ g of said protein having the ability to score at least +2 in the Rosen-modified Sampath-Reddi assay.

20. A vector comprising a DNA sequence of Claim 19 in operative association with an expression control sequence therefor.

21. A host cell transformed with a DNA sequence of Claim 19.

22. A method for producing a BMP-5 protein, said method comprising the steps of

(a) culturing in a suitable culture medium said transformed host cell of claim 21; and

(b) isolating and purifying said bone and/or cartilage inductive protein from said culture medium.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/07069

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 07 K 15/06, C 12 N 15/12, A 61 K 37/02														
II. FIELDS SEARCHED <div style="text-align: center; border: 1px solid black; padding: 2px;">Minimum Documentation Searched⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border: 1px solid black; padding: 2px;">Classification System</th> <th style="border: 1px solid black; padding: 2px;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; padding: 5px; vertical-align: top;">IPC5</td> <td style="border: 1px solid black; padding: 5px; vertical-align: top;">C 07 K; C 12 N; A 61 K</td> </tr> </table> <div style="text-align: center; border: 1px solid black; padding: 2px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched⁸</div>			Classification System	Classification Symbols	IPC5	C 07 K; C 12 N; A 61 K								
Classification System	Classification Symbols													
IPC5	C 07 K; C 12 N; A 61 K													
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 2px;">Category *</th> <th style="width: 60%; padding: 2px;">Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²</th> <th style="width: 30%; padding: 2px;">Relevant to Claim No.¹³</th> </tr> </thead> <tbody> <tr> <td style="padding: 5px; vertical-align: top;">P,X</td> <td style="padding: 5px; vertical-align: top;">WO, A1, 9011366 (GENETICS INSTITUTE, INC.) 4 October 1990, see page 45 - page 48; claim 1 --</td> <td style="padding: 5px; vertical-align: top;">1-15,17, 19-22</td> </tr> <tr> <td style="padding: 5px; vertical-align: top;">A</td> <td style="padding: 5px; vertical-align: top;">WO, A1, 8910409 (GENETICS INSTITUTE, INC.) 2 November 1989, see the whole document --</td> <td style="padding: 5px; vertical-align: top;">1-15,17, 19-22</td> </tr> <tr> <td style="padding: 5px; vertical-align: top;">A</td> <td style="padding: 5px; vertical-align: top;">WO, A1, 8800205 (GENETICS INSTITUTE, INC.) 14 January 1988, see the whole document --</td> <td style="padding: 5px; vertical-align: top;">1-15,17, 19-22</td> </tr> </tbody> </table>			Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	P,X	WO, A1, 9011366 (GENETICS INSTITUTE, INC.) 4 October 1990, see page 45 - page 48; claim 1 --	1-15,17, 19-22	A	WO, A1, 8910409 (GENETICS INSTITUTE, INC.) 2 November 1989, see the whole document --	1-15,17, 19-22	A	WO, A1, 8800205 (GENETICS INSTITUTE, INC.) 14 January 1988, see the whole document --	1-15,17, 19-22
Category *	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³												
P,X	WO, A1, 9011366 (GENETICS INSTITUTE, INC.) 4 October 1990, see page 45 - page 48; claim 1 --	1-15,17, 19-22												
A	WO, A1, 8910409 (GENETICS INSTITUTE, INC.) 2 November 1989, see the whole document --	1-15,17, 19-22												
A	WO, A1, 8800205 (GENETICS INSTITUTE, INC.) 14 January 1988, see the whole document --	1-15,17, 19-22												
<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>* Special categories of cited documents:¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px; vertical-align: top;"> Date of the Actual Completion of the International Search 20th February 1992 </td> <td style="width: 50%; border: 1px solid black; padding: 5px; vertical-align: top;"> Date of Mailing of this International Search Report 05 MAR 1992 </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px; vertical-align: top;"> International Searching Authority EUROPEAN PATENT OFFICE </td> <td style="border: 1px solid black; padding: 5px; vertical-align: top;"> Signature of Authorized Officer <div style="text-align: center;"> Mme N. KUIPER </div> </td> </tr> </table>			Date of the Actual Completion of the International Search 20th February 1992	Date of Mailing of this International Search Report 05 MAR 1992	International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer <div style="text-align: center;"> Mme N. KUIPER </div>								
Date of the Actual Completion of the International Search 20th February 1992	Date of Mailing of this International Search Report 05 MAR 1992													
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer <div style="text-align: center;"> Mme N. KUIPER </div>													

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	US, A, 4789732 (URIST) 6 December 1988, see the whole document --	1-15,17, 19-22
A	EP, A2, 0212474 (UNIVERSITY OF CALIFORNIA) 4 March 1987, see the whole document --	1-15,17, 19-22
P,X	PROC.NATL.ACAD.SCI., vol. 87, 1990, Anthony J. Celeste et al: "Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone "; figure 2 --	1-15,17, 19-22
A	Dialog Information Services, File 154, Medline 85-92 Dialog accession no. 07827756, Wozney JM: "Bone mor- phogenetic proteins", Prog Growth Factor Res 1989, 1 (4) p 267-80 -- -----	1-15,17, 19-22

V. ☒ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim numbers 16, 18 because they relate to subject matter not required to be searched by this Authority, namely:
See PCT Rule 39.1(iv)
Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods
2. ☐ Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/US 91/07069**

SA 52105

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 30/12/91
The European Patent office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9011366	04/10/90	AU-D- 5357790	22/10/90
		EP-A- 0429570	05/06/91
WO-A1- 8910409	02/11/89	AU-D- 3448789	24/11/89
		EP-A- 0408649	23/01/91
		JP-T- 3503649	15/08/91
WO-A1- 8800205	14/01/88	AU-B- 613314	01/08/91
		AU-D- 7783587	29/01/88
		EP-A- 0313578	03/05/89
		JP-T- 2500241	01/02/90
		US-A- 5013649	07/05/91
		US-A- 4877864	31/10/89
US-A- 4789732	06/12/88	US-A- 4294753	13/10/81
		US-A- 4761471	02/08/88
		US-A- 4455256	19/06/84
		US-A- 4619989	28/10/86
		US-A- 4795804	03/01/89
EP-A2- 0212474	04/03/87	JP-A- 62111933	22/05/87
		US-A- 4795804	03/01/89

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.